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Phenotypic variations and new mutations in JAK2 V617F-negative polycythemia vera, erythrocytosis and idiopathic myelofibrosis

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Abstract

(1) Objective—The chronic myeloproliferative disorders (MPD), polycythemia vera (PV), essential thrombocytosis (ET) and idiopathic myelofibrosis (IMF), are characterized by a spectrum of clinical features and linked by common genetic lesions in JAK2 and MPL. However, the clinical phenotypes in genetically undefined MPD patients are similar to those patients with JAK2 and MPL lesions. We, therefore, sought to determine whether there were JAK2 or MPL lesions in a well-defined, JAK2 V617F-negative MPD cohort, and to determine if clinical associations could be identified based on variations identified in these genes.

(2) Methods—We examined the JAK2 and MPL genes in JAK2 V617F-negative PV, IMF and idiopathic erythrocytosis (ERT) patients for sequence variations.

(3) Results—We identified two previously unrecognized JAK2 mutations and three previously unrecognized MPL mutations in JAK2 V617F-negative PV, erythrocytosis and IMF patients. We identified JAK2 exon 12 lesions in 30% of JAK2 V617F-negative PV patients, and either JAK2 V617F or JAK2 exon 12 lesions in 9% of ERT patients. In IMF, in addition to the MPL gene mutation, W515K, we identified three additional mutations: 204P and 2 intervening sequence transitions: IVS 11/12 and 10/11.

(4) Conclusions—While the clinical phenotype of JAK2 exon 12 lesions in the MPD was predominantly erythroid, there was significant disease spectrum overlap between JAK2 V617F and JAK2 exon 12 mutations. By contrast, MPL gene mutations were not associated with erythrocytosis, but segregated primarily with the phenotypes of thrombocytosis, extramedullary disease, myelofibrosis and osteosclerosis.

Introduction

Polycythemia vera (PV), idiopathic myelofibrosis (IMF) and essential thrombocytosis (ET) are clonal myeloproliferative disorders whose shared features include origin in a multipotent hematopoietic progenitor cell(1), cytogenetic abnormalities of chromosomes 1,8,9,13 and 20 (2), growth factor-independent in vitro hematopoietic colony formation(3) and epigenetic abnormalities, such as increased granulocyte PRV-1 mRNA expression(4) and impaired megakaryocyte and platelet thrombopoietin receptor protein (Mpl) expression(5). Taken together, these shared phenotypic and genotypic features suggest that PV, IMF and ET are pathogenetically related. The discovery that the same JAK2 activating mutation (V617F) was common to all three disorders supported this contention(6-9).

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However, up to 10% of patients meeting the clinical criteria for PV do not express JAK2 V617F (6). Recently, a few of these patients were found to have mutations in JAK2 exon 12(10) and the majority primarily had an erythrocytosis phenotype in contrast to the panmyelopathy that is characteristic of JAK2 V617F-positive PV patients. Importantly, in a few JAK2 V617F-negative IMF patients, new activating MPL gene mutations have been found(11-13), although the molecular basis of the bulk of these patients remains unknown.

To investigate these issues further, we examined the JAK2 gene in 157 PV patients and 44 erythrocytosis patients and sequenced the MPL gene in 32 JAK2 V617F-negative IMF patients. We report here two previously unrecognized JAK2 exon 12 mutations and 3 unrecognized MPL gene mutations together with an analysis of the corresponding genotype-phenotype correlations.

Methods

The study was approved by our Institutional Review Board, and written informed consent was obtained from all patients. The diagnosis of PV was based on the criteria of the Polycythemia Vera Study Group and included an elevated red cell mass(14). The diagnosis of IMF was based on the Italian Consensus Conference clinical criteria(15). Acquired idiopathic erythrocytosis was defined by the presence of an increased red cell mass and a low or normal serum erythropoietin level in the absence of a definable stimulus. The patients recruited to this study were evaluated in the Johns Hopkins Center for the Chronic Myeloproliferative Disorders; all blood samples were obtained between 2005 and 2007. Clinical data including cell counts, family history and spleen size were extracted from the medical record at the time blood was collected for analysis.

JAK2 sequence analysis

Genomic DNA was prepared from density gradient purified granulocytes using the Puregene Cell kit (Gentra Systems, Minneapolis). Quantitative JAK2 V617F allele percentage was performed as previously described on granulocyte genomic DNA using an allele-specific, quantitative real-time PCR assay sensitive to 10% of either the wild-type or mutant JAK2 allele (16). Exon 12 of the JAK2 gene was amplified with the following primers (Forward: 5'-CTCCTCTTTGGAGCAATTCA -3'; Reverse: 5'- AACATCTAACACAAGGTTGGCATA -3') using Qiagen Taq PCR Master Mix Kit (Qiagen, Santa Clarita CA) under the following conditions: 35 cycles of denaturation at 94° C for 1 minute, annealing at 58° C for 1 minute and extension at 72° C for 1.5 minutes. PCR products were cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). Mutations identified from abnormal electropherograms from directly sequenced PCR products were verified by cloning and plasmid sequence analysis. Sequence analysis was performed using the fluorescent dideoxy terminator method of cycle sequencing on an Applied Biosystems Division 3700 DNA analyzer.

MPL sequence analysis

MPL genomic DNA was amplified with the primers shown in Table 1 using the conditions described above. PCR products were purified from the reaction mixture using the QIAquick PCR purification kit (Qiagen, Santa Clarita CA) and sequenced as above.

Statistical Analysis

We used the t-test for continuous variables and the chi-square test for categorical variables as appropriate. P-values less than 0.05 were considered significant. Statistical calculations were performed using SigmaStat.

Results

JAK2 lesions in a JAK2 V617F-negative PV cohort

In a group of 157 subjects fulfilling the diagnostic criteria of PV, 147 (94%) were JAK2 V617F-positive. Table 2 compares the clinical features of the 10 JAK2 V617F-negative PV patients with the 147 JAK2 V617F-positive subjects. There were no significant differences in median age at presentation, disease duration, family history of an MPD, gender, antecedent history of ET, or prevalence of splenomegaly between JAK2 V617F-positive and -negative PV patients. However, in keeping with previous reports(17), both leukocyte and platelet counts were higher in the JAK2 V617F-positive patients, although only the difference in white cell count reached statistical significance in this cohort. We sequenced exon 12 of the JAK2 gene in these 10 JAK2 V617F-negative PV patients and identified two new JAK2 lesions, R541-E543delinsK and H538-K539delinsL (Figure 2), in three of them. Clinical data from these three patients are presented in Table 3A. Erythrocytosis and splenomegaly were the common denominators for clinical phenotype in this small group of patients.

JAK2 lesions in an idiopathic erythrocytosis cohort

Since the phenotype of the patients with JAK2 exon 12 lesions in this JAK2 V617F-negative PV cohort and those previously described(10) appeared to be primarily erythrocytosis, we screened 44 subjects with idiopathic erythrocytosis for both JAK2 V617F and JAK2 exon 12 lesions. We found 4 subjects harboring JAK2 mutations: 2 with JAK2 V617F (quantitative JAK2 V617F allele percentages of 34 and 59%), one with JAK2 N542-E543del and one with JAK2 R541-E543delinsK. Clinical data from these three patients are presented in Table 3B. None had any hematological abnormality except erythrocytosis.

Given the small numbers of patients identified with JAK2 exon 12 lesions, we pooled all reported patient clinical data from JAK2 exon 12 lesion subjects(10) with the clinical data from this cohort for comparison with JAK2-lesion negative erythrocytosis patients (ERT) (Table 4A) or JAK2 V617F-positive PV or erythrocytosis patients (Table 4B). Aside from a female predominance in the JAK2 lesion-positive erythrocytosis group, there were no significant differences in platelet count, white cell count, splenomegaly prevalence or age at diagnosis in the JAK2 exon 12 lesion-positive subjects from the combined cohort compared with the 40 JAK2 lesion-negative erythrocytosis patients (Table 4A). We then compared the clinical features of all the reported JAK2 exon 12 lesion patients, whether classified as PV or erythrocytosis, to all of our JAK2 V617F-positive PV or erythrocytosis patients, and found that the JAK2 V617F-positive subjects had significantly higher white cell and platelet counts compared to the JAK2 exon 12-positive subjects (Table 4B).

Clinical phenotype in a JAK2 V617F-negative IMF cohort

In a group of 32 subjects fulfilling the diagnostic criteria of IMF, 15 (47%) were JAK2 V617F-positive. Table 5 compares the clinical features of the 17 JAK2 V617F-negative IMF patients to the 15 JAK2 V617F-positive IMF patients. There were no significant differences in median age at presentation, disease duration, family history of an MPD, gender or prevalence of splenomegaly between JAK2 V617F-positive and -negative IMF patients. In both groups there was a male predominance, and there was a higher incidence of antecedent ET in the JAK2 V617F-negative group, and a significantly higher white cell count in the JAK2 V617F-positive group ($p = 0.010$).

MPL sequence variation in a JAK2 V617F-negative IMF cohort

We sequenced all the exons, the flanking intervening sequences and the 5'-proximal untranslated region of the MPL gene in the 17 JAK2 V617F-negative IMF patients presented

in Table 5. In one IMF patient, we identified a two base substitution (TG11534-5AA; Genbank accession number GI:89161185) in exon 10 the MPL gene which results in a tryptophan to lysine substitution at amino acid 515 (W515K); the wild-type sequence was not identified from either peripheral blood CD34+ cells or neutrophils isolated from this patient. In a second IMF patient, we identified a nucleotide transition (T1686C), which results in a serine to proline substitution at amino acid 240 (S204P). In two other IMF patients, we identified heterozygous nucleotide transitions in intervening sequences of the splice recognition sites of exons 10 (C11561T) and 11 (G14503A); these transitions were not identified in the public SNP databases. We directly sequenced exon 4, exon 10 and the intervening sequences of exon 10 and 11 in an additional 13 JAK2V617F-positive IMF patients and in 9 of the 10 JAK2 V617F-negative PV patients listed in Table 2; no other sequence variation was identified. Clinical data from the four IMF patients with MPL sequence variation identified is presented in Table 6 and locations of MPL sequence variation in the MPD are presented in Figure 2. Although patient numbers are small, no distinct clinical differences were characterized the JAK2 V617f-negative patients with MPL gene mutations.

Discussion

The World Health Organization (WHO) currently classifies PV, IMF and ET together with chronic myelogenous leukemia, chronic eosinophilic leukemia, chronic neutrophilic leukemia, the hypereosinophilic syndrome and unclassifiable chronic myeloproliferative disease under the rubric of the chronic myeloproliferative disorders(18). However, PV, IMF and ET have more in common phenotypically with each other than with these other disorders and this was confirmed genotypically by the discovery of the JAK2 V617F mutation(6), the expression of which is largely confined to PV, IMF and ET (19).

Extensive studies of JAK2 V617F have indicated that its frequency within PV, IMF and ET is variable, being most common in PV. The relationship of JAK2 V617F and erythrocytosis, the defining feature of PV, is supported by the observation of heightened erythropoietin sensitivity of homozygous V617F clones compared to heterozygous V617F clones(20), by higher hemoglobin concentrations in JAK2 V617F positive compared to JAK2 V617F-negative ET patients(21), by mouse gene transfection experiments(8) and more recently by the identification of JAK2 exon 12 lesions, which associate primarily with an erythrocytosis phenotype in both man and the mouse(10).

The data presented here and more extensive studies of MPL 515K/L mutations(12;13) place patients with the JAK2 exon12, and MPL 515K/L/S204P lesions together with JAK2 V617F in the spectrum of the chronic myeloproliferative disorders. While some patients with JAK2 V617F and JAK2 exon 12 lesions are clinically similar, on average the JAK2 exon 12 patients have significantly lower white cell and platelet counts than JAK2 V617F patients. In addition, JAK2 V617F, but not JAK2 exon 12 mutations, are associated with either thrombocytosis or myelofibrosis phenotypes that are clinically indistinguishable from those associated with MPL515K/L. To date, JAK2 exon 12 lesions do not appear to associate with the ET phenotype, and in only rare, longstanding cases (patient PV3, Table 3A), do they associate with significant extramedullary disease. Thus, although the number of patients currently identified with these mutations is small, so far the phenotypes generated by MPL and JAK2 exon 12 lesions do not appear to intersect, while JAK2 V617F, MPL gene mutations and Mpl epigenetic lesions do (Figure 3). When possible, a meta-analysis of larger groups of patients with JAK2 and MPL mutant alleles will be instrumental in further defining the associated clinical phenotypes.

We propose that the phenotypic variability associated with JAK2 V617F, extending from erythrocytosis alone as described here and previously(22) to idiopathic myelofibrosis, can be explained to some extent by variable JAK2 V617F allele burdens in MPD patients(16;23;24).

The JAK2 V617F allele burden alone, however, cannot entirely account for the overlap of phenotypes associated with JAK2 V617F. Beyond the JAK2 V617F clonal burden, the data suggest that other genetic or epigenetic changes in MPL and other genes are probably important modifiers of JAK2 V617F-associated disease, and MPD generating in and of themselves.

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	532	536	540	544
wildtype	ATGAACCAAATGGTGTTCACAAAATCAGAAATGAAGATTTGATATTTAAT			
	M N Q M V F H K I R N E D L I F N			
*R541-E543delinsK	ATGAACCAAATGGTGTTCACAAAATCA-----AAGATTTGATATTTAAT			
	M N Q M V F H K I K - - D L I F N			
*H538-K539delinsL	ATGAACCAAATGGTGT TTT ---AATCAGAAATGAAGATTTGATATTTAAT			
	M N Q M V F L - I R N E D L I F N			
F537-K539delinsK	ATGAACCAAATGGTG-----AAAATCAGAAATGAAGATTTGATATTTAAT			
	M N Q M V - - L I R N E D L I F N			
H538QK539L	ATGAACCAAATGGTGTTC CAATTA ATCAGAAATGAAGATTTGATATTTAAT			
	M N Q M V F Q L I R N E D L I F N			
K539L	ATGAACCAAATGGTGTTC ACTTA ATCAGAAATGAAGATTTGATATTTAAT			
	M N Q M V F H L I R N E D L I F N			
N542-E543del	ATGAACCAAATGGTGTTCACAAAATCAGA-----GATTTGATATTTAAT			
	M N Q M V F H K I R - - D L I F N			
* new mutations				

Figure 1.

Somatic mutations of JAK2 exon 12 identified in JAK2 V617F-negative PV and idiopathic erythrocytosis patients. Unique mutations identified from this cohort are indicated by the asterisks. Mutations not described in this study are from Scott et al(10).

Figure 2. MPL sequence variation associated with the chronic myeloproliferative disorders, K39N(25), S505N(26), W515K/L(11;12) as previously described.

Figure 3.
Spectrum of MPD phenotypes associated with JAK2 and MPL lesions

Table 1

Primer pairs to amplify MPL regions

Target Exon	Forward (5' to 3')	Reverse (5' to 3')
5' flank B	AGCCATCTCCAATCTGAGCA	GAGATGAGGAAACGGAGATACA
5' flank A	CAGCAGATTAGCCTCCCAAG	CATCTTCTCCGCCACTGTGT
1-3	CTGAAGGGAGGATGGGCTA	GTGACAGGAGGATGGCTCTT
4	AGTCCAGAGGCTGAGCCATA	AGGTCTGGAATCCCCAAAGT
5-6	CCACAGAGATGCTGTGCAAAAT	TTCTGCAAGATTGTGGCTCA
7-8	AGGCCATCGTTCTTGTAGGA	TCCCAAAGACAAAGGGAAGA
9-10	GGATTGGGTCAAACAGACG	GCGGTATAGTGGGCGTGTTA
11-12	CCATGGCTCAGTCTGCTTCT	ACTGGGAGTGAGGAGCCTGT

Table 2
Clinical features of JAK2 V617F-positive and –negative PV patients.

	JAK2 V617F-positive N= 147	JAK2 V617F-negative N= 10
% female	61%	60%
Median age at diagnosis (range)	49 (18-74)	50 (18-67)
Median disease duration (years, range)	8 (1-28)	6 (3-54)
Antecedent ET history	21%	20%
Family MPD history	8%	20%
Palpable splenomegaly	43%	50%
White cell count (K/cu mm)	11.9 (3.6-98.4) *	8.1 (4.5-20.2) *
Platelet count (K/cu mm)	507 (18-1320) **	427 (87-682) **

*
p <0.001

**
NS

Table 3

Clinical features of patients with JAK2 mutations

A. Subjects classified as PV						
Patient	JAK2 lesion	Age at diagnosis/Gender	Disease duration (years)	White cell count (K/cu mm)	Platelet count (K/cu mm)	Palpable splenomegaly
PV1	R541-E543delinsK	61/M	1	6.7	579	1 cm
PV2	R541-E543delinsK	50/F	5	7.8	303	2 cm
PV3	H538-K539delinsL	18/M	54	11.0	60	massive

B. Subjects classified as idiopathic erythrocytosis						
Patient	JAK2 lesion	Age at diagnosis/Gender	Disease duration (years)	White cell count (K/cu mm)	Platelet count (K/cu mm)	Palpable splenomegaly
ERT1	N542-E543del	54/F	15	8.5	257	none
ERT2	R541-E543delinsK	15/M	1	5.8	291	none
ERT3	JAK2 V617F	41/F	1	5.7	227	none
ERT4	JAK2 V617F	82/F	10	6.9	111	none

Table 4

Clinical features of patients with erythrocytosis

A. Subjects classified as idiopathic erythrocytosis		
	JAK2 lesion-negative ERT N= 40	JAK2 lesion-positive ERT[*] N= 8
% female	35%	75%
Median age at diagnosis (range)	49 (18-74)	41(15-82)
Palpable splenomegaly	0%	0%
White cell count (K/cu mm)	6.9 (4.2-14.7)	6.4 (5.1-8.6)
Platelet count (K/cu mm)	227 (109-753)	285 (111-308)
B. Subjects with erythrocytosis and a JAK2 lesion		
	JAK2 V617F-positive PV or ERT N= 149	JAK2 exon12 lesions PV or ERT N= 15[*]
% female	61%	53%
Median age at diagnosis (range)	49 (18-74)	50 (15-62)
Palpable splenomegaly	43%	40%
White cell count (K/cu mm)	11.9 (3.6-98.4) [§]	7.8 (6.7-12.1) [§]
Platelet count (K/cu mm)	507 (18-1320) [#]	298 (60-579) [#]

* includes erythrocytosis patients from Scott et al(10).

* includes PV and erythrocytosis patients from Scott et al(10)

[§] p=0.001

[#] p=0.017

Table 5

Clinical features of JAK2 V617F-positive and –negative IMF patients.

	JAK2 V617F-positive N= 15	JAK2 V617F-negative N= 17
% male	60%	71%
Median age at diagnosis (range)	59 (38-74)	56 (26-71)
Median disease duration (years, range)	8 (0-20)	5 (1-28)
Antecedent ET history	20%	41%
Family MPD history	8%	0%
Palpable splenomegaly	100%	94%
White cell count (K/cu mm)	15.3 (4.17-93.2) *	7.4 (1.7-51.9) *
Hemoglobin (g/dl)	11.7 (6.9-14.0)	10.2 (6.8-13.2)
Platelet count (K/cu mm)	198 (167-402) **	260 (3-1074) **

*
p = 0.010**
NS

Table 6

Clinical features of IMF patients with MPL sequence variation

Patient	MPL lesion	Age at diagnosis/ Gender	Disease duration (years)	White cell count (K/cu mm)	Hemoglobin (g/ dl)	Platelet count (K/ cu mm)	Bone marrow	Palpable splenomegaly
IMF1	W515K	26/M	23	8.6 [*]	12.1	209	osteosclerosis	Massive
IMF2	S204P	42/M	1	11.1 [*]	12.8	368	osteosclerosis	Moderate
IMF3	IVS 11/12	71/F	3	51.9 [*]	8.8	24	osteosclerosis	Massive
IMF4	IVS 10/11	35/M	31	2.8 [*]	6.8	47	osteosclerosis	Massive

^{*} White cell differential left-shifted to the myelocyte stage